



Department of Chemistry,
University of Puerto Rico, Río
Piedras Campus, PO Box 23346,
San Juan, PR 00931-3346, USA

Karen G. Carrasquillo, Juan
Carlos Aponte Carro, Adriana
Alejandro, Diana Diaz Toro,
Kai Griebenow

Correspondence: K. Griebenow,
Department of Chemistry,
University of Puerto Rico, Río
Piedras Campus, PO Box 23346,
San Juan, PR 00931-3346, USA.

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Reduction of structural perturbations in bovine serum albumin by non-aqueous microencapsulation

Karen G. Carrasquillo, Juan Carlos Aponte Carro, Adriana Alejandro,
Diana Diaz Toro, Kai Griebenow

Abstract

Protein stability is a factor limiting the use of sustained-release devices in medical applications. The aim of this study was to reduce structural perturbations occurring in the frequently used model protein, bovine serum albumin (BSA), upon microencapsulation in poly(D,L-lactide-co-glycolide) (PLG) microspheres.

Spray freeze-dried BSA was encapsulated into PLG microspheres by a completely non-aqueous oil-in-oil encapsulation procedure. FTIR spectroscopy was used as a non-invasive method to quantify procedure-induced structural perturbations in BSA. Spray-freeze drying of BSA caused significant structural perturbations that were minimized by co-spray freeze-drying BSA with trehalose. BSA-containing microspheres were produced by suspension of the powder by homogenization in methylene chloride containing PLG, followed by formation of coacervate droplets by the addition of silicon oil and hardening using the solvent heptane. Resulting microspheres had dimensions of approximately 100 μm and the encapsulation efficiency for BSA was > 90%. FTIR data showed that the structure of the BSA–trehalose formulation encapsulated into PLG microspheres was less perturbed than that of BSA obtained from buffer alone.

The results demonstrate that the structure-guided encapsulation approach introduced for non-aqueous casting encapsulation procedures can be extended to the non-aqueous production of pharmaceutically relevant PLG microspheres involving a complex encapsulation procedure.

Introduction

Much effort is currently devoted to developing new or improving existing techniques to encapsulate protein drugs into biodegradable polymers (Schwendeman et al 1996; Johansen et al 2000). However, few studies address the question of how the encapsulation procedure impacts on the protein structure (Griebenow et al 1999), despite the fact that this has repeatedly been identified to be of importance to improve delivery devices (Schwendeman et al 1996; Thomasin et al 1998). It is reasonable to assume that minimization of protein structural perturbations during their encapsulation into biodegradable polymers will lead to the delivery of unmodified, as opposed to, for example, aggregated and thus potentially immunogenic, protein pharmaceuticals. This concept has recently been shown to hold true for the accelerated storage stability of recombinant human growth hormone (rhGH) (Costantino et al 1998).

One promising approach to encapsulate proteins into biocompatible polymers involves the use of completely non-aqueous protocols using dehydrated amorphous protein powders. It has been established that the conformational mobility is drastically reduced in such powders (Griebenow et al 1999). Thus, amorphous protein powders can be suspended in various neat organic solvents without causing solvent-induced protein structural perturbations (Griebenow & Klibanov 1996). This fact has been utilized in non-aqueous casting encapsulation protocols to minimize structural perturbations for bovine serum albumin (BSA) (Carrasquillo et al 1998) and rhGH (Carrasquillo et al 1999). Using FTIR spectroscopy as a non-invasive spectroscopic method allowing characterization of the secondary structure of the model proteins at various stages of encapsulation, procedure-induced structural perturbations were systematically eradicated. The most common procedure used for the encapsulation of proteins into PLG microspheres, the so-called double emulsion-solvent evaporation or water-in-oil-in-water (w/o/w) technique fails to accomplish this objective (Schwendeman et al 1996; Fu et al 1999). It has become increasingly apparent that one major problem with protein encapsulation by the w/o/w technique involves the formation of the first emulsion (Schwendeman et al 1996; Griebenow et al 1999). Here, an aqueous protein solution is introduced into an organic solvent (typically methylene chloride or ethyl acetate) containing the dissolved polymer. The emulsion is formed by a short sonication or homogenization procedure. Proteins tend to accumulate at the solvent-water interface and are prone to denaturation and aggregation (Sah 1999). Incomplete delivery of proteins from delivery devices has frequently been related to such procedure-induced protein aggregation (Tabata et al 1993; Alonso et al 1994; Lu & Park 1995).

In this study, we have developed a completely non-aqueous oil-in-oil coacervation protocol for the formation of BSA-loaded PLG microspheres. FTIR spectroscopy was used to non-invasively monitor BSA secondary structure at relevant steps of the micro-encapsulation and within the PLG microspheres. We present the first evidence for protein structural preservation upon microsphere preparation and thus extend the feasibility studies (Carrasquillo et al 1998, 1999) to a medically relevant delivery system.

Materials and Methods

Materials

BSA ($\geq 96\%$ albumin, essentially fatty acid free) and trehalose were obtained from Sigma. Dichloromethane (99.9%, ACS HPLC grade) and heptane (99%, anhydrous) were from Aldrich. Poly(dimethylsiloxane) (silicone oil) was from Lancaster Synthesis. Poly(D,L-lactide-co-glycolide) with a copolymer ratio of 50:50 (resomer RG502H) was from Boehringer Mannheim.

Spray freeze-drying of BSA

The first step in non-aqueous encapsulation protocols is to obtain a dehydrated protein formulation by a suitable method, for example, freeze-drying (Griebenow et al 1999). Freeze-dried BSA powder (Carrasquillo et al 1998) could not be used efficiently in the oil-in-oil encapsulation procedure because we noted a low encapsulation efficiency ($< 15\%$) and microsphere yield ($< 20\%$), probably caused by the large particle size (50–100 μm) observed by scanning electron microscopy (SEM) (Maa & Hsu 1997). Thus we obtained spray freeze-dried BSA powder that had a more sponge-like appearance. Such powders are easily disintegrated into very small particles in the first suspension step (Costantino et al 2000) of the oil-in-oil encapsulation procedure, prerequisite for high encapsulation efficiency and yield (Maa & Hsu 1997).

Aqueous solutions containing 10 mg mL⁻¹ BSA with or without trehalose at a 1:4 mass ratio (protein/trehalose) were atomized with N₂ gas through a nozzle with a 558.8- μm orifice (Paasche Airbrush, VLS-set) into liquid N₂. The N₂ gas pressure was adjusted manually to approximately 10–15 psi. The frozen protein solution was collected into a pre-cooled glass beaker and stored in a freezer at -20°C for 3 h to evaporate the liquid N₂. The frozen protein particles were then lyophilized using a Labconco Freezone 6L lyophilizer at a condenser temperature of -45°C and a pressure of $< 60 \mu\text{mHg}$, for 48 h, sealed in 20-mL scintillation glass vials, and stored at $2-8^\circ\text{C}$.

Microsphere preparation

BSA (40 mg) was suspended in methylene chloride containing 200 mg mL⁻¹ of dissolved PLG using a VirTis Tempest homogenizer equipped with a 10-mm shaft at approximately 20000 rev min⁻¹ (set speed 4) for 1 min. After protein suspension, homogenization intensity was lowered to 15000 rev min⁻¹ (set speed 3) and the

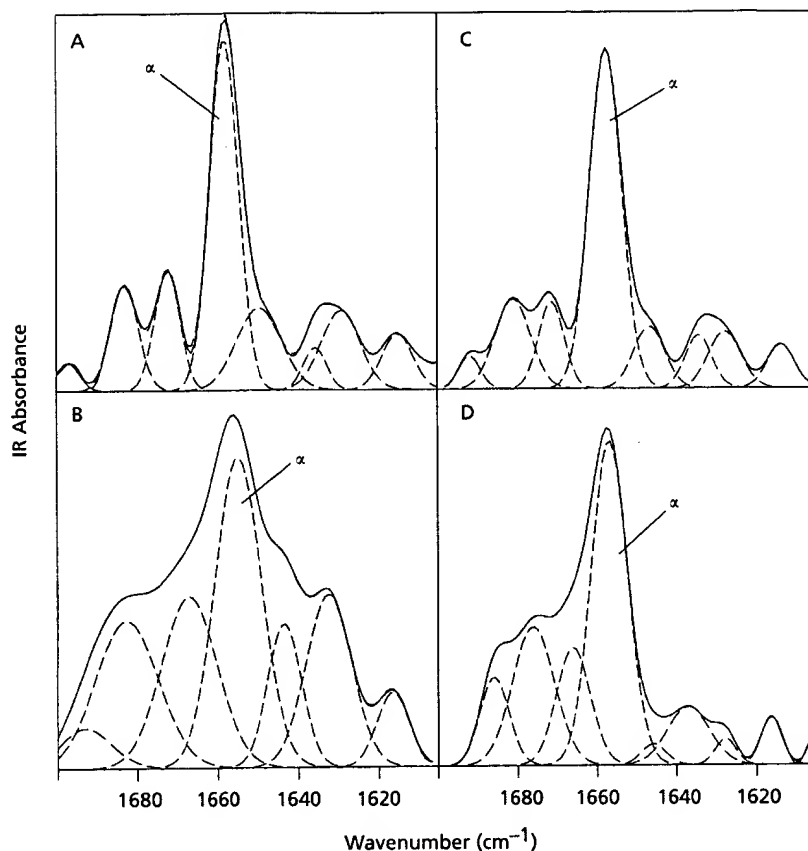


Figure 1 Resolution-enhanced amide I FTIR spectra and Gaussian curve-fitting of: BSA in aqueous solution (A); BSA powder obtained by spray freeze-drying (B); BSA powder obtained by co-spray freeze-drying with trehalose at a 1:4 mass ratio (C); and BSA powder obtained by co-spray freeze-drying with trehalose and encapsulated in PLG microspheres (D). The solid lines are the resolution enhanced amide I FTIR spectra overlaid with the result of the Gaussian curve-fitting (both practically coincide), the broken lines are the individual Gaussian bands fitted to the spectra. The Gaussian band assigned to α -helices is marked in the spectra.

coacervating agent poly(dimethylsiloxane) was added at a rate of 2 mL min^{-1} . The coacervating mixture containing the microspheres was then poured into an Erlenmeyer flask containing 100 mL heptane under constant agitation and stirred for 2 h to allow hardening of the microspheres. Microspheres were collected by filtration using a $0.22\text{-}\mu\text{m}$ pore size nylon filter, washed twice with heptane, and dried for 24 h at $< 60 \text{ }\mu\text{mHg}$. The procedure was optimized for high encapsulation efficiency by variation of the homogenization intensity. Protein-loading of the microspheres was determined according to Tabata et al (1993). Protein concentration was determined from its absorbance at 280 nm (Tabata et al 1993). The encapsulation efficiency (%BSA encapsulated in PLG microspheres) was determined as described by Atkins & Peacock (1996).

SEM

SEM of microspheres was performed using a Jeol 5800 LV. Samples were coated with gold ($200\text{--}500 \text{ \AA}$) using a Denton Vacuum DV-502A.

Protein secondary structural analysis

FTIR spectra were acquired using a Magna-IR System 560 optical bench and corrected for excipients, PLG background, and water vapour as previously described (Carrasquillo et al 1998, 1999). The secondary structure of BSA was quantified by Gaussian curve-fitting of the resolution-enhanced amide I band ($1700\text{--}1615 \text{ cm}^{-1}$) also as previously described (Carrasquillo et al 1998).

Table 1 Secondary structure content of BSA spray freeze-dried under different conditions.

Sample/State	Secondary structure content (%)		
	α -Helix	β -Sheet	Other
BSA with no additives			
Aqueous solution	54 \pm 6	8 \pm 3	38 \pm 1
Spray freeze-dried powder	34 \pm 2	19 \pm 2	47 \pm 2
Microencapsulated in PLG	31 \pm 4	18 \pm 2	51 \pm 3
BSA spray freeze-dried with trehalose			
Spray freeze-dried powder	52 \pm 1	3 \pm 2	45 \pm 2
Microencapsulated in PLG	42 \pm 1	3 \pm 1	55 \pm 2

BSA was co-spray freeze-dried with trehalose at a 1:4 mass ratio. The secondary structure was calculated by Gaussian curve-fitting of the amide I spectra after band-narrowing by Fourier self-deconvolution. Other structural elements include random coil, extended chains, and turns.

In-vitro release profile

Microspheres (10 mg) were placed in 2 mL 10 mM phosphate buffer adjusted to pH 7.3, and incubated at 37°C. Every 24 h, microspheres were centrifuged for 1 min at 500 rev min⁻¹. The supernatant was removed and the protein concentration determined. Microspheres were resuspended in 2 mL of fresh buffer to maintain sink conditions. Protein concentration values were used to construct cumulative release profiles. Release experiments were performed in triplicate, the results averaged, and the standard deviations calculated.

Results and Discussion

The structure of spray freeze-dried BSA was assessed by FTIR spectroscopy (Figure 1B). Comparison with the spectrum of native BSA in aqueous solution at pH 7.3 (Figure 1A), shows a significant broadening of the amide I spectral components indicating a significant loss of order in the individual elements of the secondary structure as the result of the drying procedure. Quantification of the secondary structure by Gaussian curve-fitting of the resolution-enhanced amide I FTIR spectra (Carrasquillo et al 1998) revealed a decrease in the α -helix content from 54 to 34%, and an increase in the β -sheet content from 8 to 19% upon spray freeze-drying of BSA (Table 1). Co-spray freeze-drying of BSA with trehalose reduced these structural perturbations. The amide I spectrum obtained for the co-spray freeze-dried preparation (Figure 1C) was more similar to that of BSA in aqueous solution (Figure 1A), than to that without the additive (Figure 1B). The secondary struc-

ture content was calculated and was within the same error as that for BSA in aqueous solution (Table 1).

Both spray freeze-dried BSA formulations were encapsulated into PLG microspheres. Optimization of the procedure yielded conditions leading to an encapsulation efficiency of > 90% for both formulations, and protein FTIR spectra could be obtained. For both preparations, the follow-up encapsulation procedure caused some structural perturbations in BSA, which are highlighted for the encapsulated BSA-trehalose formulation in Figure 1D. Compared with the spectrum of the formulation before encapsulation (Figure 1C), some increase in intensity for components between approximately 1660 and 1690 cm⁻¹ was evident (Figure 1D). Components in this spectral range are usually assigned to amide I vibrational modes of various turns (e.g. β -turn) (Griebenow et al 1999). Otherwise, only small changes in the amide I band shape were visible. A decrease in the α -helix content from 52 to 42% and an increase in other structural elements from 45 to 55% were observed (Table 1). However, the spectrum of the encapsulated BSA-trehalose formulation (Figure 1D) was more similar to the spectrum of BSA in aqueous solution (Figure 1A) than to that of spray freeze-dried BSA (Figure 1B), or that of encapsulated spray freeze-dried BSA (spectrum not shown). This is supported by quantitative data (Table 1). Although the α -helix content for the BSA-trehalose formulation encapsulated in PLG (42%) was less than that for BSA in aqueous solution (54%), it was substantially higher than that for spray freeze-dried BSA encapsulated in PLG (31%). The main conclusion from this is that when the structural changes in BSA induced by spray freeze-drying were minimized using trehalose, the structure of

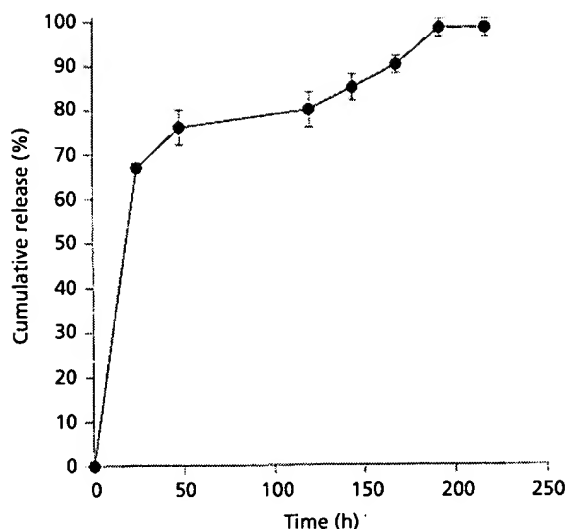


Figure 2 Cumulative release of spray freeze-dried BSA-trehalose from PLG microspheres produced by oil-in-oil encapsulation.

BSA within the PLG microspheres was more native. However, further work is necessary to develop strategies that minimize the encapsulation-induced structural perturbations.

PLG microspheres containing spray freeze-dried BSA were further characterized. Previous experiments using suspended solid protein powders for the formation of microspheres resulted in an initial burst release of approximately 60–70 % of the protein within the first 24 h (Bittner et al 1998; Schwendeman et al 1998). This has been attributed to incomplete protein encapsulation and/or swelling of the core material. In-vitro release experiments with the PLG microspheres obtained in this study revealed an initial burst release of > 60 % (Figure 2). The burst release in this case was likely caused by the formation of porous microspheres. SEM images show that the microspheres obtained were heterogeneous in size (average diam. approx. 128 μm for BSA, and approx. 60 μm for BSA-trehalose PLG microspheres), somewhat irregularly shaped, and with visible pores on the surface (Figure 3). However, the in-vitro release profile showed that BSA release was complete (Figure 2). Thus, no insoluble BSA aggregates were formed during encapsulation or release.

Conclusions

In this study we have addressed the issue of protein structure within PLG microspheres. Our results support the view that the non-aqueous encapsulation method-

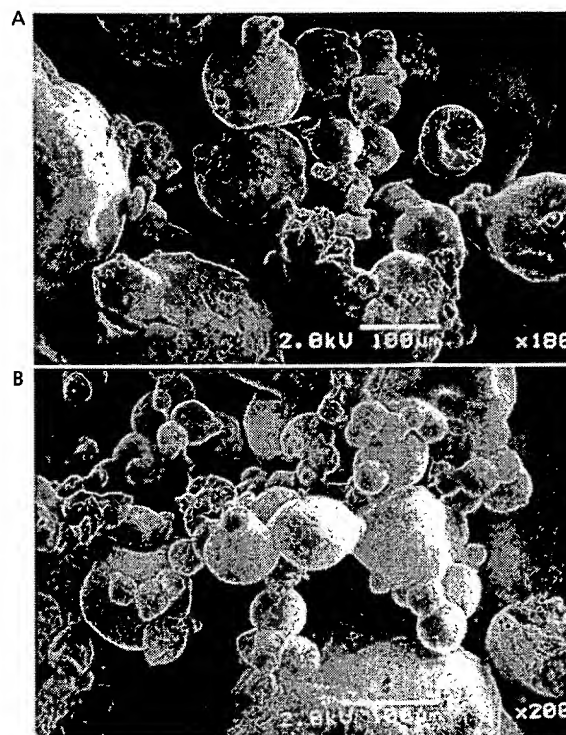


Figure 3 SEM images of PLG microspheres containing spray freeze-dried BSA (A), and spray freeze-dried BSA-trehalose (B).

ology introduced for slab polymer geometries (Carrasquillo et al 1998, 1999), can be extended to non-aqueous oil-in-oil coacervation encapsulation procedures. This study highlights the potential benefit of using suspended protein powders under non-aqueous conditions for the formation of polymer microspheres. However, some draw-backs have also become evident, in particular, the substantial initial burst release. Further, methodologies have to be developed that prevent encapsulation-induced protein structural perturbations.

Results and Discussion

- Alonso, M. J., Gupta, R. K., Min, C., Siber, G. R., Langer, R. (1994) Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. *Vaccine* 12: 299–306
- Atkins, T. W., Peacock, S. J. (1996) The incorporation and release of bovine serum albumin from poly-hydroxybutyrate-hydroxyvalerate microcapsules. *J. Microencapsul.* 13: 709–717

- Bittner, B., Ronneberger, B., Zange, R., Volland, C., Anderson, J. M., Kissel, T. (1998) Bovine serum albumin loaded microspheres: the influence of polymer purity on particle characteristics. *J. Microencapsul.* **15**: 495–514
- Carrasquillo, K. G., Cordero, R. A., Ho, S., Franquiz, J. M., Griebenow, K. (1998) Structure-guided encapsulation of bovine serum albumin in poly(DL-lactic-co-glycolic) acid. *Pharm. Pharmacol. Commun.* **4**: 563–571
- Carrasquillo, K. G., Costantino, H. R., Cordero, R. A., Hsu, C. C., Griebenow, K. (1999) On the structural preservation of recombinant human growth hormone in a dried film of a synthetic biodegradable polymer. *J. Pharm. Sci.* **88**: 166–173
- Costantino, H. R., Carrasquillo, K. G., Cordero, R. A., Mummenthaler, M., Hsu, C. C., Griebenow, K. (1998) The effect of excipients on the structure and stability of lyophilized recombinant human growth hormone (rhGH). *J. Pharm. Sci.* **87**: 1412–1420
- Costantino, H. R., Firouzabadian, L., Hogeland, K., Wu, C.-C., Beganski, C., Carrasquillo, K. G., Córdova, M., Griebenow, K., Zale, S. E., Tracy, M. (2000) Protein spray freeze drying. Effect of atomization conditions on particle size and stability. *Pharm. Res.* In press
- Fu, K., Griebenow, K., Hsieh, L., Klibanov, A. M., Langer, R. (1999) FTIR characterization of the secondary structure of proteins encapsulated within PLGA microspheres. *J. Contr. Rel.* **19**: 357–366
- Griebenow, K., Klibanov, A. M. (1996) On protein denaturation in aqueous-organic but not in pure organic solvents. *J. Am. Chem. Soc.* **118**: 11695–11700
- Griebenow, K., Castellanos, I. J., Carrasquillo, K. G. (1999) Application of FTIR spectroscopy to probe and improve protein structure in sustained release devices. *Internet J. Vibr. Spec.* [www.ijvs.com] **3**, 5, 2
- Johansen, P., Men, Y., Merkle, H. P., Gander, B. (2000) Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination. *Eur. J. Pharm. Biopharm.* **50**: 129–146
- Lu, W., Park, T. G. (1995) In vitro release profiles of eristatin from biodegradable polymeric microspheres: protein aggregation problem. *Biotechnol. Prog.* **11**: 224–227
- Maa, Y.-F., Hsu, C. C. (1997) Effect of primary emulsions on microsphere size and protein-loading in the double emulsion process. *J. Microencapsul.* **14**: 225–241
- Sah, H. (1999) Protein behavior at the water/methylene chloride interface. *J. Pharm. Sci.* **88**: 1320–1325
- Schwendeman, S. P., Cardamone, M., Brandon, M. R., Klibanov, A. M., Langer, R. (1996) Stability of proteins and their delivery from biodegradable polymer microspheres. In: Cohen, S., Bernstein, H. (eds) *Microparticulate Systems for the Delivery of Proteins and Vaccines*. Marcel Dekker, New York, pp 1–49
- Schwendeman, S. P., Tobio, M., Joworowicz, M., Alonso, M. J., Langer, R. (1998) New strategies for the microencapsulation of tetanus vaccine. *J. Microencapsul.* **15**: 299–318
- Tabata, Y., Gutta, S., Langer, R. (1993) Controlled delivery systems for proteins using polyanhydride microspheres. *Pharm. Res.* **10**: 487–496
- Thomasin, C., Merkle, H. P., Gander, B. (1998) Drug microencapsulation by PLA/PLGA coacervation in the light of thermodynamics. I. Overview and theoretical considerations. *J. Pharm. Sci.* **87**: 259–268